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Spermatophore dimorphism in the chokka squid *Loligo reynaudii* associated with
alternative mating tactics

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Short running head: DIMORPHISM IN AFRICAN SQUID

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Abstract

Chokka squid *Loligo reynaudii* have characteristic alternative mating tactics - large “consort” males form a temporal pair with a female and transfer spermatophores to within the female’s mantle cavity. On the other hand, small “sneaker” males rush towards a pair and transfer spermatophores onto the female’s buccal membrane. The differences in mating behaviour and the related sperm storage sites place clear constraints on the fertilization process and can drive divergent adaptation between consort and sneaker males. However, morphological adaptation associated with alternative mating tactics has not been examined in this species. We observed the morphology of spermatangium (sperm mass ejaculated from the spermatophore capsule) from each sperm storage site on the female body, and found dimorphism in spermatangium associated with the alternative sperm storage sites. We also observed spermatophores stored in the Needham’s sac of mature males and confirmed that small males produced “sneaker-type” spermatangium. Therefore mating tactics adopted by individual males is distinguishable without a requirement for direct behavioural observation. The information helps for better understanding of the reproductive system and the population structure in this species.

Introduction

Sexual selection works on male traits not only before mating to obtain females but also after mating to ensure fertilization (Parker 1970). Sperm competition theory predicts that males strategically allocate sperm in each mating, and males with high sperm competition risk use more sperm in a single mating (Parker 1993). Males having well developed secondary sexual traits are advantaged in the competition to obtain females, and are able to win favorable mating positions and order. On the other hand, males having less developed secondary sexual traits have to reconcile to a less favorable mating position and order. Such asymmetry in competitiveness can lead to alternative mating tactics and associated male dimorphism within a species (e.g. Gross 1996, Emlen 1997, Oliveira et al. 2008). Alternative mating tactics lead to asymmetry in sperm competition risk, and males may adapt their sperm allocation pattern and sperm quality (Parker 1990, Snook 2005).

Alternative mating tactics are commonly observed in loliginid squid in the genera *Loligo* and *Doryteuthis* (e.g. Hanlon & Messenger 1996, Sauer et al. 1997, Hanlon et al. 2002, Buresch et al. 2009, Iwata et al. 2005, Marian 2012). Males in these species show body size dimorphism. Large “consort” males compete with rival males, form a temporal pair with a female, transfer spermatophores into the female’s mantle cavity, and guard the

female until spawning. Alternatively, small “sneaker” males usually do not fight with other males but rush toward a pair and transfer spermatophores onto the female’s buccal membrane without mate guarding. These alternative mating tactics cause multiple paternities in a brood, and lead to biased fertilization success among males (Hanlon et al. 1997, Shaw & Sauer 2004, Iwata et al. 2005, Buresch et al. 2009, Naud et al. 2016). Differences in mating position and spermatophore placement results in differences in the fertilization environment and in sperm competition, and can be a strong sexual selection force. Diverged morphological and physiological adaptations associated with alternative mating tactics has been described in detail in the loliginid squid *Heterololigo bleekeri* – large consort males produce long spermatophores, rope-like spermatangia, and small sperm with short longevity, alternatively small sneaker males produce short spermatophores, drop-like spermatangia, and large sperm with long longevity and characteristic chemotaxis leading to self-aggregation (Iwata & Sakurai 2007, Iwata et al. 2011, Hirohashi et al. 2013, Iwata et al. 2015, Hirohashi et al. 2016). Similar dimorphism is found in *Doryteuthis plei* (Marian 2012, Apostólico & Marian 2017), suggesting that such distinct morphological and physiological adaptation associated with alternative mating tactics would be common in this group.

Similar to the loliginid squid mentioned above, chokka squid (*Loligo reynaudii*) has

alternative mating tactics related with body size dimorphism (Sauer et al. 1997, Sauer et al. 2000, Smale et al. 2001), and shows multiple paternity in siblings (Shaw & Sauer 2004, Naud et al. 2016). Sperm competition and the different fertilization conditions (external vs. internal) may also lead to dimorphism in sperm transfer pattern in this species. Furthermore, the fishery on *L. reynaudii* targets spawning aggregations by jigging, and the fishing method tends to selectively catch large males (Lipinski 1994). Managing such fisheries necessitates a detailed understanding of the reproductive ecology of the species to allow more accurate predictions of possible changes to the population demography caused by selective fishing pressure. However, the morphological characteristics of consort males and small sneaker males are unknown, as previous studies on the mating system were based on *in situ* behavioral observations by SCUBA, and the information is limited due to the difficult diving conditions at 25-50 m depth (Sauer et al. 1997, Sauer et al. 2000, Smale et al. 2001, Shaw & Sauer 2004, Naud et al. 2016). In this study, we analyzed the presence of spermatophore dimorphism associated with alternative mating tactics in *L. reynaudii* to identify individual mating tactics from a morphological perspective, allowing better understanding of the population structure.

Materials and Methods

A total of 145 female and 287 male *Loligo reynaudii* were collected by jigging on the spawning grounds in St. Francis bay (Figure 1) in November 2008 - 2010. All individuals were kept at 4 °C after landing and dissected within two days.

For each female, two sites of spermatophore attachment (oviduct in mantle cavity and buccal membrane) were examined for the presence of attached spermatangia (Fig. 2A).

If there were attached spermatangia, their morphology was observed under a stereo microscope. The presence of stored sperm in the seminal receptacle was detected on the basis of a swollen seminal receptacle with a distinctive white colour.

All males, except one individual, were fully mature. Sixty of the 286 mature males had only broken spermatophores in the Needham's sac, therefore we used 226 males to distinguish the spermatophore morphology. The mantle length of each individual male and the length of ten spermatophores randomly collected from Needham's sac were measured with electric slide calipers. In addition, spermatophores were removed from the Needham's sac by forceps, placed on a petri dish filled with filtered seawater, and stimulated to induce a spermatophoric reaction. The morphology of ejaculated spermatangia were subsequently observed under a stereomicroscope.

Results

Of 145 females examined, all females were fully mature and had sperm in the seminal receptacle. Spermatangia were found around the seminal receptacle of 104 females (71.7%), on the oviduct of 30 females (20.7%) and both sites of 25 females (17.2%).

For the 104 females with spermatangia placed around the seminal receptacle, two females had only cement bodies of the spermatangia remaining and the rest had drop-like spermatangia (Fig. 2B). The oviduct had a wide membrane near the opening, and spermatangia were found attached to this (Fig. 2C). Spermatangia had a cement body which has an adhesive material on the head. Since 5 of 30 females (16.6%) with spermatangia on the oviduct had only cement bodies of spermatangia remaining attached, the morphology of individual spermatangia was not possible to elucidate. In the remaining 25 females, 24 females had only rope-type spermatangia and one female had only drop-type spermatangia on the oviduct (Fig. 2D).

The mantle length of mature males ranged from 115 mm to 428 mm (N = 287). Spermatophore size was related with body size, and larger males had longer spermatophores (Fig.3). Spermatangium dimorphism was observed among males - 160 males had rope-type spermatangia and 68 males had drop-type spermatangia (Fig. 3). Large males (mantle length 147 - 428 mm) had rope-type spermatangia and small males

(mantle length 115 - 200 mm) had drop-type spermatangia. Both morphological types were observed in the range of mantle lengths 147 - 200 mm ($N = 98$, drop type: 56 individuals, rope-type: 42 individuals). 12 of the 98 individuals did not have intact spermatophores in the storage organs, however it was possible to classify them through the morphology of spermatangia accidentally ejaculated in the storage organ; however it was not possible to measure their spermatophore length. Therefore, we compared spermatophore length of the remaining 86 males throughout the body length range (drop type: 51 individuals, rope-type 35 individuals). Spermatophores producing rope-type spermatangium were longer than spermatophores producing drop-type spermatangium (ANCOVA, $F_{(1, 82)} = 70.1$, $P < 0.001$).

Combining these results we were able to define small males producing drop-like spermatangia as sneaker males attaching spermatophores around the seminal receptacle, and large males producing rope-like spermatangia as consort males attaching spermatophores on the oviduct. We performed a logistic regression analysis with the morphology of the spermatangium as the dependent variable, and mantle length as a covariate. The result indicated a significant relationship between spermatangium morphology and mantle length (logistic regression model: $\chi^2_2 = 167.79$, $P < 0.001$; mantle length: $z = 6.037$, $P < 0.001$, Fig. 4), with an estimated mantle length of 175.9mm for

which there was a 50 % probability of a sneaker.

Among consort males, it appears that the relationship between mantle length and spermatophore length change at some point (Fig. 3). Therefore, we tested for dimorphism among consort males using the method of Eberhard & Guitiérrez (1991). According to the method, we fitted a partial regression equation to determine whether the relationship between mantle length (ML) and spermatophore length (SL) was nonlinear, which would indicate a potential dimorphism (Iwata & Sakurai 2007, Apostólico & Marian 2017). Results confirmed a dimorphism with the slope of the relationship changing at a mantle length of 273 mm ($ML < 273 \text{ mm}$: $SL = 0.044 ML + 4.165$, $ML > 273 \text{ mm}$: $SL = 0.022ML + 10.213$; $R^2 = 0.932$, $P < 0.001$). The allometric slope was notably more gentle in the larger consort males than the smaller consort males, suggesting that large males allocate less resources to a single spermatophore. Spermatophore length was significantly correlated with mantle length in sneaker males ($SL = 0.035 ML + 4.554$, $R^2 = 0.517$, $P < 0.001$).

Discussion

Alternative mating tactics are well known phenomenon in various taxa (Oliveira et al. 2008), and in cephalopods have been recorded in loliginid squids (Drew 1919, Hanlon

1996, Hanlon et al. 1997, Hanlon et al. 2002, Jantzen & Havenhand 2003, Wada et al. 2005) and a cuttlefish (Norman et al. 1999, Hall & Hanlon 2002). Alternative mating tactics show a clear contrast in sperm competition risk, and this can lead to strategic sperm release among males (Parker 1990). Loliginid squid also reveal a rare characteristic having discrete sperm transfer sites for each mating tactic, one inside the mantle cavity for consort males and another around the mouth for sneaker males (Drew 1911). These discrete sperm transfer sites show a clear difference in sperm competition risk and fertilization environment, and have led to morphological and physiological adaptations for each situation (Iwata & Sakurai 2007, Iwata et al. 2011, Iwata et al. 2015, Hirohashi et al. 2013, Hirohashi et al. 2016a, Hirohashi et al. 2016b, Marian 2012, Apostólico & Marian 2017). Dimorphism in spermatophore and spermatangium has been reported previously in two species of loliginidae, *Heteriloligo bleekeri* distributed in East Asia (Iwata & Sakurai 2007, Iwata et al. 2015) and *Doryteuthis plei* distributed in South Atlantic Ocean (Apostólico & Marian 2017). Spermatophore size relates to the sperm allocation strategy associated with sperm competition risk for each mating tactic. The number of spermatozoa within a single spermatophore is 5-folds greater in consort males than sneaker males in *H. bleekeri* (Iwata et al. 2011), and sperm mass volume is greater in consorts than in sneakers in *D. plei* (Apostrolico & Marian 2017). Similarly in this

study, spermatophore size and spermatangium shape dimorphisms were found in *Loligo reynaudii*, distributed off the South African coast, suggesting that dimorphism associated with alternative mating tactics is a common phenomenon in loliginid squid. In previous studies, males were classified into a tactic type according to the switch point in the relationship between spermatophore length and mantle length (Iwata & Sakurai 2007, Apostólico & Marian 2017). However, the relationship in *L. reynaudii* is continuous, and body size distribution of sneaker and consort males widely overlapped. Therefore this infers that the classification according to the relationship between spermatophore length and body size is not suitable in this case. Spermatophores in loliginid squid have dimorphism not only in the spermatophore size but also in the morphology of the spermatangia (Iwata et al. 2015). Spermatangia morphology was found to be discrete with two distinct shapes in *L. reynaudii*, and we therefore, classified individuals according to spermatangia morphology.

The oviduct opening of females in *Loligo reynaudii* had a wide membrane lip, and spermatophores transferred into the mantle cavity by male-parallel copulations were attached on the surface of the membrane in all cases, suggesting that the membrane works as a receptor for spermatophores. In *Heterololigo bleekeri*, consort males attach spermatophores on the inside wall of the oviduct, and the oviduct opening does not have

such a membrane lip (Iwata and Sakurai 2007). However, a similar membrane structure had been observed in *Dorytheuthis plei*, and males in this species attach spermatophores on the membrane (Marian 2012). The membrane lip in these two species is not similar to a seminal receptacle, which is designed to keep spermatozoa alive for a long duration, and is likely to function as a receptor for spermatangia for short periods only, during active spawning events. Our results, showing 16.6 % of females with spermatangia on the oviduct opening had only the cement body remaining, further supports this view.

Dorytheuthis plei revealed two morphological switch points in the relationship between spermatophore size and body size, one switch point related to smaller body size, dividing sneaker and consort males, and the other related to the size range of consort males, suggesting three alternative mating tactics in this species (Apostólico and Marian 2017). In *L. reynaudii* and *D. plei*, the relationship between spermatophore length and mantle length is continuous but the slope becomes gentler in large consort males. The largest males allocate relatively small resources to a single spermatophore, suggesting that they have a lower sperm competition risk. Existence of three mating tactics is known in isopoda (Shuster & Wade 1991) and some lizards (Sinervo & Lively 1996). In those cases, mating tactics of males are genetically determined (Shuster & Sassaman 1997, Sinervo & Zamudio 2001), and the three tactics are evolutionary stable, dictated by the

mechanism of alternative strategies, where all tactics bring equal fitness (Shuster & Wade 1991) or a frequency dependent selection, not dissimilar to rock-paper-scissors game situation (Sinervo & Zamudio 2001). In such a genetically determined mating system, one would expect the tactic adopted by each male to be fixed both morphologically and behaviorally. On the other hand, it is known that there are three types of males in reindeer *Rangifer tarandus*, harem-holders, sneakers, and mixed-tactic males using both tactics (harem-holding and sneaking) according to circumstances (Pintus et al. 2015). In the oval squid *Sepioteuthis lessoniana*, males exhibit three distinct mating behaviours: male-parallel, male-upturned and sneaking, although the mating position by the sneaker is identical to the male-upturned, therefore in fact there are only two sperm transfer sites: one on the oviduct opening for male-parallel mating and the other on the buccal membrane for male-upturned and sneaking (Wada et al. 2005). Oval squid males sometimes change their mating tactics according to the relative body size of rival males. Although behavioural data is lacking, it might be possible that males with intermediate body size in *L. reynaudii* use both mating tactics flexibly according to the situation during male-male competition and female choice – e.g. when the competing male or target female is larger, the male may switch mating behavior toward sneaking. In fact our results did show sneaker type spermatangia attached to the oviduct opening, although it was only

one in 25 cases, suggesting that a male's mating tactic is not necessarily fixed but males changing tactics is unusual.

An alternate hypothesis would be a presence of a further unknown mating tactic, which utilizes the male-parallel position but with a different sperm competition risk. In male peacock wrasse *Symphodus tinca*, there are four types of mating tactics – “nest owners” who build a nest with seaweed and guard the nest and spawned egg, “satellites” who have a small body size and spawn by rushing into the other's nest whenever a female spawns, “interceptors” who have a wide range of body sizes and intercept females to spawn, away from a nest where no parental care would be provided, and “pirates” who are the largest males which parasitize the effort of nesting males by fighting with nest owners and taking over high quality nests, and then abandon the nest to the original owner after the spawning peak (van den Berghe 1988). Although *L. reynaudii* do not build a nest or provide parental care after spawning, consort males guard females during spawning (Hanlon et al. 2002). Paternity analysis revealed that consort males sometimes did not sire any offspring (21% of broods sampled), suggesting that consort males intercepted females guarded by other consort males, and in fact such displacement of consort males is not rare (Naud et al. 2016). The largest consort males producing relatively small spermatophores may behave as pirates, who pay less effort to guard females and simply take over females just before

spawning, with less sperm competition risk. The reason for the presence of a switch point in the relationship between mantle length and spermatophore length among consort males is still elusive and further morphological and behavioral observations are necessary to fully understand the mating systems for this species.

In conclusion, this study revealed discrete dimorphism in spermatangia morphology associated with two alternative sperm transfer sites on the female's body in the Southern African chokka squid *Loligo reynaudii*. According to the morphological difference, males can be classified as consorts or sneakers. Our results also suggested a possibility of the presence two types of consort males in sperm allocation strategy. Such information is useful for better understanding the reproductive system and the population structure in this species.

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Figure captions

Figure 1. Sampling sites for *Loligo reynaudii* during a commercial closed fishing season in November 2008-2010.

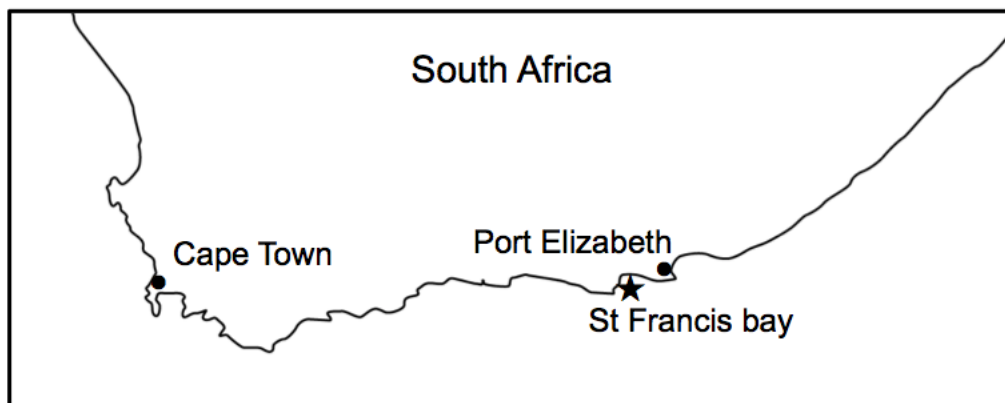


Figure 2. Spermatangia attached on the female body. A) Sperm transfer sites (white arrowhead) in mantle cavity by consort males through male-parallel mating. B) Spermatangia (white arrowheads) attached around seminal receptacle (black arrowhead). C) Attached spermatangia (white arrowhead) on the membrane lip of oviduct opening. D) Wide membrane on the oviduct opening (white arrowhead).

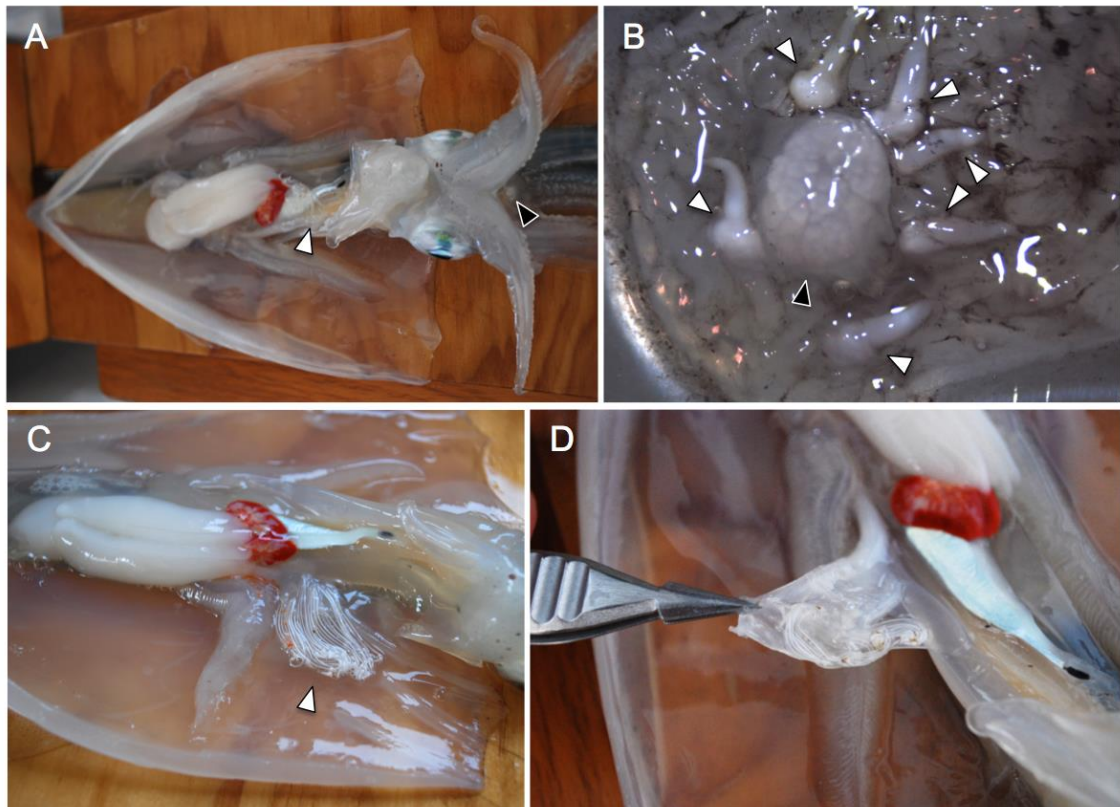


Figure 3. Relationship between mantle length and spermatophore length. Males having rope-type spermatangia indicated by a closed circle and drop-type by an open circle.

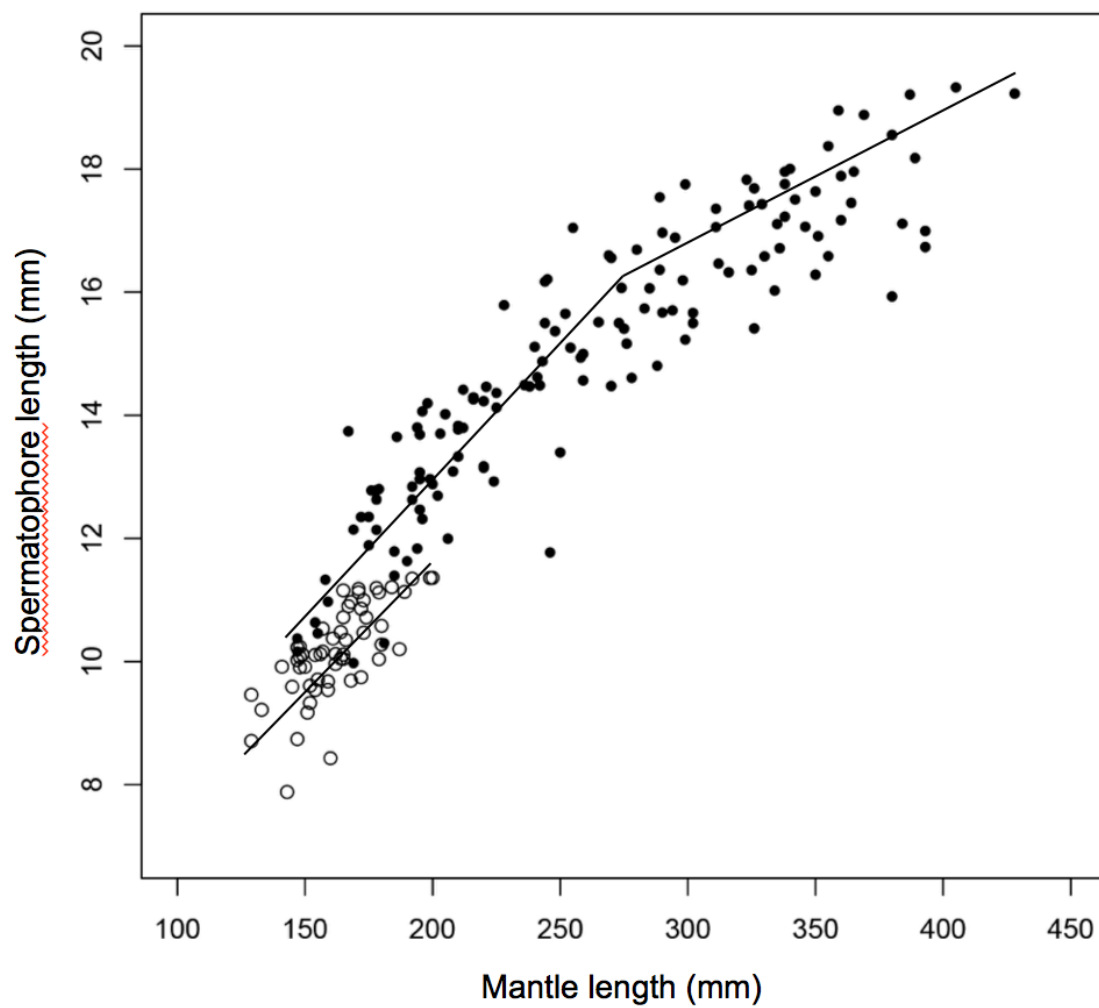


Figure 4. Logistic regression between mantle length and the possibility of a consort male

(sneaker = 0, consort = 1).

